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Department of Biosciences

Surface attachment dynamics of the rotifer *Brachionus plicatilis* under different surface to volume ratio.

Master thesis

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Abstract

In order to describe prey-predator relationships, several models suggest functional response is dependent on prey density alone, while challenging theories support that the ratio between prey and predator density is more relevant. Predation models have extendedly been investigated using various model organisms, rotifers included. However recent investigations have proposed that available surfaces seem to have a significant impact on the ecology of the free swimming rotifer *Branchionus plicatilis*. Females of this species have a pedal gland on their foot that allows them to deliberately attach to surface habitats, a strategy which has proven to be beneficiary for maximum net growth. To investigate the dynamical properties of the surface habitat microcosm systems were set into non-layered and multilayered flasks, using *Tetraselmis sp.* as prey and *Branchionus plicatilis* as predator. I conducted two experiments where a significant effect of surface available on the abundance of rotifers was revealed, confirming that surfaces are quality habitats for rotifers. Additionally a novel application of freeze drying was used in order to monitor algal biomass development and compared to the traditional filtering method. A simple linear model was formulated in order to test whether explicit predictions from the results of this study pointed towards prey or ratio dependence. Data from *Experiment 1* are partially consistent with a ratio dependent functional response model. On the contrary, considering that replicates in *Experiment 2* had not reached a steady equilibrium state, it can be concluded that data from *Experiment 2* point towards a prey-dependent functional response. The effects of temporary attachment of rotifers to surfaces thus seem to be important and linked to the availability of nutrients in their habitat.

Acknowledgments

This master thesis was carried out at the University of Oslo. The thesis has been written under the guidance of Professor Josefin Titelman and Professor Tom Andersen.

Upon the completion of this thesis I would like to express my gratitude to Professor Josefin Titelman for invaluable supervision, motivating pep talks and always being available for discussions and questions and Professor Tom Andersen for helping me plan my experiments and for providing excellent guidance and valuable comments during the analytic work. Also I would like to thank Jan-Eric Thrane for advices and assistance on the pigment extraction methods. Loads of gratitude to the AKVA technicians: Sissel Brubak, Per-Johan Færøvig and Rita Amudsen for their valuable help during the experimental work. I would like express my sincere thanks to Catharina Broch for cooperation and encouragement along the way. I would like to express my heartfelt thanks all students at the AKVA department for helpful discussions and for all social gatherings and activities that made me feel a part of the student life of the university. Finally I would like to take this opportunity to express my deep gratitude to my family for their persistent support and encouragement.

CONTENTS

Introduction

Natural communities are structured by a network of interactions among species and of the species with their environment. Among the most important are trophic interactions, known as consumer-resource interactions or prey-predator relationships. Over the past century many researchers have proposed different mathematical models while trying to describe these relationships.

Predation models

A Historical Retrospection

Development of ecosystem models has become an increasingly active area of research in ecology the past decades. An essential feature in ecology, the predator – prey equations were initially proposed by Alfred J. Lotka in 1910. Sixteen years later V. Volterra independently investigated these equations. From there on these equations was a major contribution to population and behavioral ecology, until 1959 when C.S. Holling extended this model yet again.

Holling (1959) developed the notion of functional response, the relationship between prey density and the rate at which prey is consumed. As ecosystem models began to be applied to environmental problems, it increasingly became important to further investigate the mathematical properties of individual functions in the models. Thirty years later, Arditi and Ginzburg (1989) challenged Holling's model, and proposed some controversial modifications that have led to the production of many articles by various authors. Many of the expressions proposed have been subjected to detailed testing and analysis in order to provide a clear understanding of the implications of the mathematical formulation of the models.

In ecology, functional response models explain the change in predation rate by an average individual predator in response to a change in density of the prey (Holling 1959). The functional response largely determines the dynamic stability of a system. Additionally it reflects the responses to environmental influences and the nature of indirect effects in the food web containing the predator–prey pair (Abrams and Ginzburg 2000).

In association with the functional response, the numerical response is defined as the change in predator density as a function of change in prey density. Overall predation can be expressed as a combination of both functional and numerical responses (Holling 1959). However, the numerical response is not necessarily proportional to the change in prey density, but usually involves a time lag between prey and predator populations (Ricklefs 2008).

Many different formulations of the trophic function have been proposed. These try to include dependence on prey density alone, the ratio of prey and predator densities, or prey and predator densities separately. Broadly speaking, these formulations are expressed with models falling under three categories: prey-dependent, ratio-dependent and intermediate (Table 1). Such models indicate striking differences in food chains of increasing length in response to variations in primary productivity (Arditi and Ginzburg 1989).

An approach following the classical Lotka-Volterra tradition is that the functional response, depends solely on prey density (Rosenzweig 1971). In this version of a prey-dependent model, both the functional and numerical responses by predators to changes in prey density are monotonically increasing and decelerating, and are often modeled by a rectangular hyperbola (Type-II response, see Figure 1) (Holling 1966). This decelerating intake rate is based on the assumptions that the consumer is limited only by its capacity to process food and that food search and handling are mutually exclusive behaviors. However, at sufficiently high productivity this two-species equilibrium becomes unstable. Then again, the

classical assumptions that predators encounter prey at random and that the trophic function depends on prey abundance only should be considered when formulating a model (Arditi and Ginzburg 1989). Therefore this expression of the trophic function is based on the assumption that there is no interference among predators and their density does not affect predation rate directly.

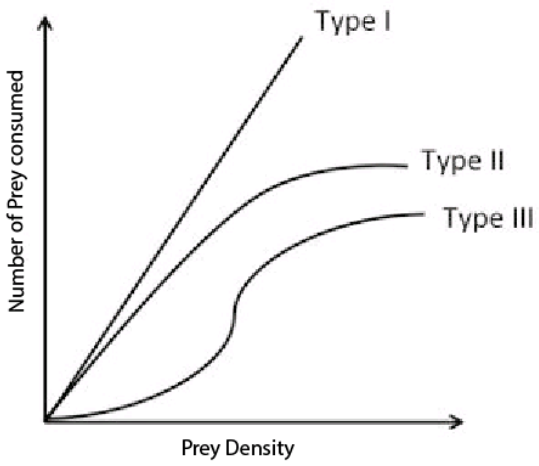

Figure 1. Three types of functional response curves. Adapted from Holling (1959).

In fact, several studies point out that in most cases predation rate decreases as the abundance of predators increases, because they have to share the same resource among a larger number of predators (Arditi et al. 1991, Abrams and Ginzburg 2000). Meanwhile, in the absence of predators, prey density is increasing linearly as carrying capacity of prey is increasing. Prey-dependent models generally do not predict that consecutive levels of both prey and predator abundance will increase alongside in a state of equilibrium just as in response to an increase in productivity. Hence, improved environmental conditions should not necessarily increase prey and predator density and could even lead to a stochastic extinction of both predators and prey as claimed by a theory called “paradox of enrichment” (Rosenzweig 1971).

In contrast, the ratio-dependent models, predict a steady increase in both prey and predator density as productivity increases (Ginzburg and Akçakaya 1992). Moreover, all levels respond proportionately, while in the prey-dependent model the responses differ depending on the trophic level and on the number of levels. Arditi et al. (1991) proposed that measuring the functional response on a longer time scale, would in most cases introduce the above mentioned mechanisms that decrease the predation. As a result, the rate of predation and the predator population growth rate of are generally considered as important functions of the *per-capita* resources of the predator.

Functions of the intermediate type models include those first proposed by DeAngelis et al. (1975). Intermediate type models try to develop a coupling link between the two, introducing parameters which measure the degree of interference among predators while consuming resources. Arditi and Akçakaya (1990) showed that in most cases the interference parameter is significant and the functional responses would depend on the strength of this interference.

In this project, I experimentally examined the population growth of rotifers *Branchionus plicatilis* under different resource (nutrients, habitat) regimes and tried to interpret the results in the context of ratio dependent and prey density dependent models. A prey dependence model would be predict that the density of prey would be constant for all different types of treatments, while a ratio dependence model would suggest proportionality between numbers of prey and predators (Table 1).

Table 1. Examples of prey-dependent, intermediate, and ratio-dependent trophic functions g(N,P,), *N = number of prey; P = number of predators; a, b, c are constants

Type	Name	Equation*	Source
Prey-dependent	Lotka-Volterra	$g(N,P) = aN$	Volterra (1928)
Prey-dependent	Holling	$g(N,P) = \frac{aN}{(b+N)}$	Holling (1959)
Ratio-dependent	Arditi-Ginzburg	$g(N,P) = g\left(\frac{N}{P}\right)$	Arditi & Ginsburg (1989)
Intermediate	DeAngelis	$g(N,P) = \frac{aN}{b+N+cP}$	DeAngelis (1975)

Role and Importance

First described by Antoni van Leeuwenhoek in the late 1600s, rotifers, or "wheel animals", are pseudocoelomate metazoans belonging to the phylum Rotifera, which comprises over two thousand species (Wallace et al. 1991, Wallace et al. 2006, Segers 2007). Their role in an ecosystem's production is of considerable importance (Armengol et al. 2001). Rotifers are characterized by their rapid population growth, their reproductive strategy and susceptibility (Bennett and Boraas 1989). They are widely distributed in freshwater and marine habitats; they also live in the soil, in mosses, and associated with lichens on rocks and trees (Hutchinson 1967). For inhabiting such a wide range for habitats and because of that they are easy to grow, rotifers are well suited for ecological and evolutionary studies (Fussmann 2011).

Parthenogenesis on Rotifers

Rotifers can have unusual population structures, due to their particular life cycle, with cyclical parthenogenesis. Cyclical parthenogenesis consists of a more or less regular alternation of sexual and asexual reproduction (Gilbert 1975) (Figure 2). Parthenogenesis may result in rapid population growth and allows for colonization of a habitat, when conditions are favorable, while offering the advantage of long term survival through resting egg production, when conditions deteriorate. Additionally, the linkage between dormancy and sexual reproduction allows long term-survival or rotifer populations (Carmona et al. 2009).Moreover, it eliminates the problem of mating encounters and the cost of producing males, allowing an asexual population to grow faster than a sexual one (Serra et al. 2004).

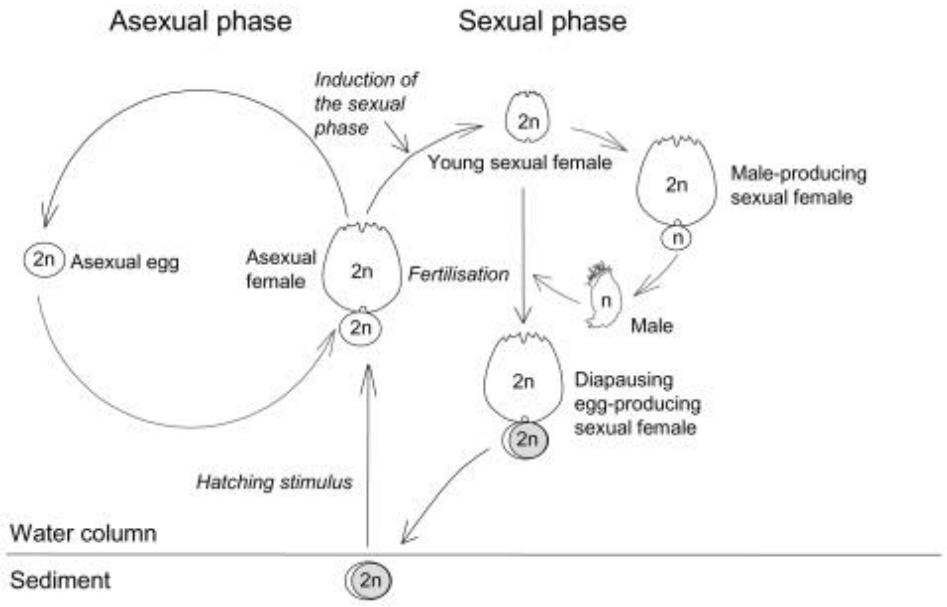

Figure 2 Rotifer reproduction cycles (picture from Hoff, 1987)

In cyclically parthenogenetic taxa such as rotifers, populations are usually initiated from sexually derived diapausing eggs (Hales et al. 1997). After a long period of dormancy and upon receiving a hatching stimulus, the diapausing eggs (dormant female embryos) will hatch into amictic females and the parthenogenetic cycle will start once again. All hatchlings from sexual eggs are asexual females that reproduce by parthenogenesis (Figure 2, Figure 3B). After the parthenogenetic phase, asexual females produce males which are able to fertilize mature females(Ruttner-Kolisko 1974). The appearance of both sexes is often associated with habitat deterioration (Carvalho and Wolf 1989). Therefore the occurrence of cyclical parthenogenesis poses a unique opportunity to use these animals as an experimental organism.

Anatomy

Rotifers are bilaterally symmetrical, unsegmented, varying in shape, size (~50–2000 μm) and hardness. Their body has a fluid-filled body cavity called pseudocoelom. The hydrostatic pressure of the pseudocoelom gives the body a supportive framework that acts as a skeleton. Rotifers are distinguished by a ciliated anterior corona which is used in food gathering, as well as a muscular pharynx equipped with a complex set of jaws called trophi. Furthermore their body wall

(integument) is thickened by special proteins that regulate thickness and flexibility of the animal (Figure 3A, 3B), (Wallace and Smith 2001).

Some species, like *Brachionus plicatilis*, have a foot that extends ventrally from the body. It may be very short or quite long (e.g. twice the body length) and it may possess several projections called "toes". In some free swimming rotifers the foot houses pedal glands, which secrete an adhesive that permits a temporary attachment to surfaces in free-swimming rotifers (Figure 3A). However, in sessile rotifers the adhesive is much stronger; if dislodged, a sessile rotifer cannot reattach (Wallace 1980).

The females of the rotifer *Brachionus*, the genus used within this thesis, have such adhesive glands on their foot and are able to temporarily attach to surfaces (Gilbert 1963). While attached they continue acquiring food as filter feeders by moving their corona. They can deliberately switch from a free-swimming to an attached mode depending on the food concentration levels in their habitat (Vadstein et al. 2012).

Brachionus plicatilis

The rotifer species *Brachionus plicatilis* is an ecological generalist, with a cosmopolitan distribution in coastal marine and inland habitats (Figure 3). However, recent studies have uncovered an issue in its taxonomy. Differences in genetic markers, ecological preferences, mixis responses and reproductive strategies have led researchers to characterize it as a cryptic species complex that consists of three different strains (Gómez and Snell 1996, Ciros-Pérez et al. 2001). *Brachionus plicatilis* has been the subject of extensive research due to its wide spread use in aquaculture as live food for marine fish larvae (Lubzens et al. 1989), thus making it the only commercially important rotifer. In addition it could serve a number of possible applications in various fields such as environmental control of eutrophication and harmful algal blooms, management of pollution and petroleum compounds, wastewater treatment and impact of climate change on biodiversity (Kostopoulou et al. 2012).

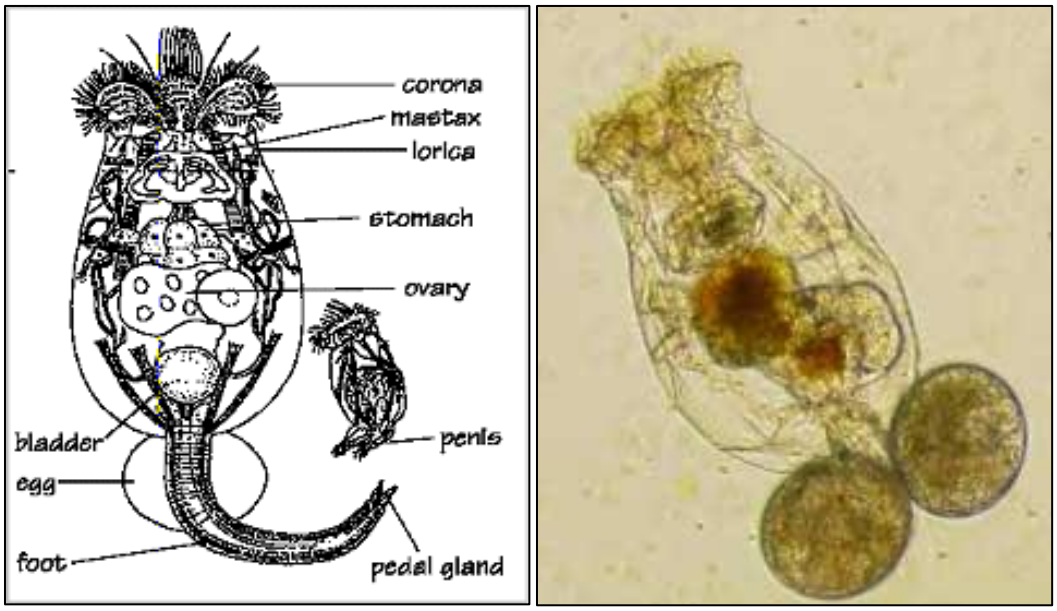

Figure 3. A. *Branchionus Plicatilis* (from Walker 1981) B. Female individual with eggs (from aquatax.de)

Surface attachment dynamics

Attachment to surfaces is the most favorable situation for suspension feeders (Breznak et al. 1984, Fenchel 1987). When attached, aquatic animals, may maximize net energy gain by avoiding the high energetics cost of swimming and by more efficient prey utilization due to reduced viscous drag (Epp and Lewis Jr 1984, Fenchel 1987). Moreover, available surfaces seem to have a significant impact on the ecology of the species *Brachionus plicatilis*, as this mainly pelagic species also inhabits temporal and permanent littoral ponds (García-Roger et al. 2006). The submerged vegetation in these ponds, serves as temporal attachment surfaces (Pennak 1955). The potential for attachment in junction with their anatomy and feeding behavior raise questions about whether the abundance of suitable surface habitats influences the population dynamics of *Brachionus plicatilis*. Although the wall habitat is the energetically most profitable, it is also limited in size compared to the open water habitat.

The use of surfaces as refuge from predators has received much attention (Jessup et al. 2005). However, for *Brachionus plicatilis*, the utilization of surface as a resource remains largely unexplored [But see Vadstein, Olsen et al. (2012)]. Surfaces where the rotifers can attach provide a high-quality habitat, which may result in a predator-dependent functional response (Vadstein et al. 2012) Also, attachment of rotifers to surfaces is dynamic and density dependent, and it apparently affected intrinsic growth rates under conditions with surplus of food (Vadstein et al. 2012)

Aim

The overall aim of my master project was to investigate the potential benefits of the use of surface as a resource for rotifer growth. To examine this I exposed rotifers to habitats differing in surface to volume ratios under different nutrient regimes. I used rotifers of the genus *Branchionus plicatilis "Nevada"* due to their ability to deliberately switch from a free-swimming to an attached state, depending on the food concentration levels in their habitat.

Based on Vadstein, Olsen et al. (2012) I predicted and tested the hypothesis that increasing surface to volume ratio would increase the equilibrium of algal biomass and rotifer abundance at all trophic levels, according the ratio dependence hypothesis. I used a gradient of limiting nutrients, in order to investigate whether the effect of surfaces is amplified by carrying capacity of prey.

To monitor the algal biomass development in my experiments, I used two different techniques for chlorophyll *a* (chl-*a*) extraction: i) the traditional filtering method and ii) a novel application of the freeze drying method, that had not previously been used to study salt water samples (details in methods). A secondary aim of the project was to test the liability of the freeze drying method.

Materials and methods

I conducted two experiments targeted at examining the role of surface to volume ratio of habitats for rotifer growth dynamics, while exposed to different nutrient availability.

Experimental organisms & cultures:

Prey

As prey I used a strain of the prasinophyte microalgae *Tetraselmis sp*. (10 µm long x 14 µm wide) (Figure 4) originally obtained from the "Stazione Zoologica A. Dohrn" in Napoli by Jahn Throndsen in 1986. It has since been kept in continuous culture at the University of Oslo.

Figure 4 *Tetraselmis sp.* (Photo provided by the Algae Resource Database)

Tetraselmis may occur as a flagellate or a non-motile cell attached by a gelatinous stalk. Flagellate cells have four flagella emerging from the pit in two pairs. A distinctive wall (theca) composed of small scale like particles in a crystalline array covers cells. Motile cells often stop swimming for extended periods, and flagella are sometimes lost. Though usually green, some can become red by accumulation of carotenoids. Various species are reported from marine and freshwaters (Sym and Pienaar 1993)

A pre-experimental, low-density, phosphate rich and semi-continuous batch culture of *Tetraselmis sp*. was maintained for several months under constant temperature 14°C. The culture medium was IMR ½ mineral nutrient medium (Eppley et al. 1967) [containing macro and micro mineral nutrient, and vitamins;], based on milli-Q water mixed to 20ppt salinity with aquarium salt (Crystal Sea by Marine Enterprises International), aerated and later sterile filtered (0.2µm pore size).

Predators

The rotifer *Branchionus plicatilis* was used as predator, and specifically a strain called *Branchionus plicatilis 'Nevada'* , which belongs to the species complex (length 250–270µm) (Gómez et al. 2002) (Figure 3B). It was kindly provided by Olav Vadstein.

I set up a culture from a parthenogenetic strain feeding on *Teraselmis*, and was kept for several generations prior to being used as predator in my experiments. All rotifer cultures were kept in a temperature controlled room at 19°C, with constant light cycle of 18:6 hours (light: darkness). Microscopic examination confirmed no contamination by other species. Prior to the experiments, rotifer cultures were gently screened through a 50µm mesh to remove algae from the culture. The rotifers were re-suspended in fresh algal solution of the nutrient concentration to be tested, allowing animals to adapt to experimental conditions for 1 day, so that all experiments were performed with animals in the same good physiological state

EXPERIMENTAL SETUP:

I conducted 2 experiments, both examining the role of habitat as a resource for *Branchionus plicatilis* growth, while in a gradient of prey (*Tetraselmis sp.*) availability. In both experiments the surface to volume ratio of the habitat was achieved by using culture flasks with different number of layers (0-3-5) (Figure 5). The carrying capacity of prey organisms was modified by adjusting the phosphate concentration of the growth medium.

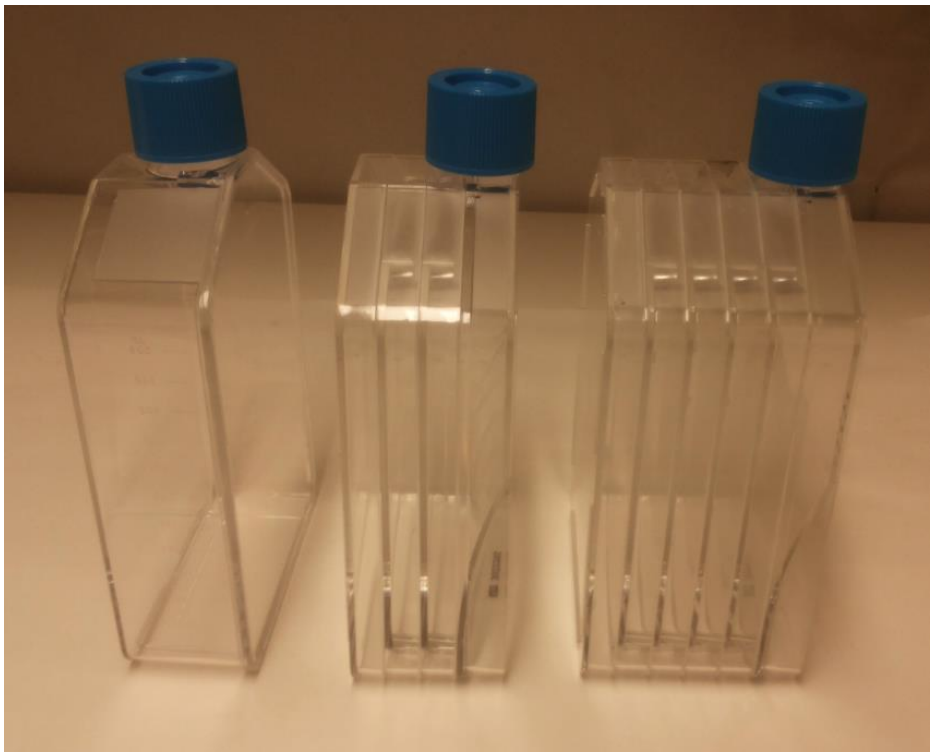

Figure 5 The three different types of flasks used as microcosms. (0, 3, 5 layered BD Falcon Cell Culture Multi-Flasks).

Both experiments were conducted under the same environmental conditions as the rotifer culturing, i.e. at 19^{o}C (±1°C) and a light dark cycle of 18:6 hours and lasted for 30 days. The experimental set up is summarized in Tables 2, 3 and detailed below.

Experiment 1

Experiment 1 had a straight forward two factor design testing the effect of surface to volume ratios of habitats and nutrient availability on rotifer growth. Each factor had three levels. For each of the 9 treatment combinations I used 5 replicates, equaling a total of 45 flasks (Table 2). The growth response was measured firstly as chl-*a* concentration every second day, and as rotifer abundance at the end of the experiment (see "*Sampling and Analysis*").

Three different types of multilayered flasks were used as microcosms, in order to achieve a wide range of surface to volume ratios. The three levels of nutrients concentration was achieved by adjusting phosphorus concentrations (0.5, 1 and 2μM) using a reduced phosphate version of IMR ½ Culture medium (Table 3).

At the beginning of the experiment, I measured algal concentration in my original algae culture using a CASY particle counter (Model TTC, Cell Counter and Analyzer, Roche Life Sciences). Then I inoculated the same number of cells to each of the 3 different phosphorus level media to get the desired starting concentration ($4*10^5$ cells$\times L^{-1}$) and filled the culture flasks up to half. Rotifers were added individually to the flasks to a concentration of ~35 adult individuals$\times L^{-1}$ in a random flask order. Subsequently the flasks were topped and gently mixed. I placed them sideways under a light source (100 μmol×photons$\times m^{-2}\times s^{-1}$, measured using biospherical Instruments QSL-100), providing light above and on the side of the flasks. The microcosms were diluted with fresh media (0.5, 1 and 2 μM) manually, every other day at a standard, dilution rate of $0.01d^{-1}$. To keep alga in suspension the flasks were gently turned once daily. To ensure that flasks were not subjected to location dependent subtle differences in environmental conditions, they were rotated to a new position once a day according to a preset scheme every day.

Experiment 2

This experiment was designed to further investigate the results of *Experiment 1*. *Experiment 2* had a similar two factor design, but with two levels of the factors this time. I used non-layered and 3-layered flasks for the surface to volume gradient and only 0.5 and 2 μM phosphate media. For each of the 4 treatment combinations I used 5 replicates, equaling a total of 20 flasks. The experiment ran for 30 days, as results from *Experiment 1* suggested that to be sufficient to reach steady state dynamics (Figure 12). This time rotifers and algae were sampled every two days and two different methods were used to quantify the algae.

As in *Experiment 1*, I first measured the algae concentration. I diluted into the low phosphate medium to the algae start concentration of 6×10^4 cells$\times L^{-1}$. Next, I filled the flasks with the adjusted phosphate algal cultures. For each replicate I picked 25 adult rotifers and placed those in Petri dishes with some filtered saltwater. These were then poured into the flasks in a random order. After that I topped the flasks by the rest of particular medium and deposited them sideways under the light source. The microcosms were diluted with fresh media (0.5 and 2 μM) manually, every other day at a dilution rate of $0.01 d^{-1}$.

Finally after the final sampling for chl-*a* all bottles were fixed in Lugol's solution to a final concentration of 1 % for later enumeration of rotifers.

Table 2. First Experiment Treatments Setup

Treatment type	Bottle Surface (cm^2)	Bottle Volume (cm^3)	Ratio S/V (cm^{-1})	$[NaH_2PO_4]$ ($\mu mol \times L^{-1}$)	Starting Algæ ($cells \times L^{-1}$)
0-layers Low Phosphate	175	750	0.633	0.5	4×10^5
3-layers Low Phosphate	525	750	2.090	0.5	4×10^5
5-layers Medium Phosphate	875	1200	1.910	0.5	4×10^5
0-layers Medium Phosphate	175	750	0.633	1.0	4×10^5
3-layers Medium Phosphate	525	750	2.090	1.0	4×10^5
5-layers Medium Phosphate	875	1200	1.910	1.0	4×10^5
0-layers High Phosphate	175	750	0.633	2.0	4×10^5
3-layers High Phosphate	525	750	2.090	2.0	4×10^5
5-layers High Phosphate	875	1200	1.910	2.0	4×10^5

Table 3. Second Experiment Treatments Setup

Treatment type	Bottle Surface (cm^2)	Bottle Volume (cm^3)	S/V Ratio (cm^{-1})	$[NaH_2PO_4]$ ($\mu mol \times L^{-1}$)	Starting Algæ ($cells \times L^{-1}$)
0-layers Low Phosphate	175	750	0.633	0.5	6×10^4
3-layers Low Phosphate	525	750	2.100	0.5	6×10^4
0-layers High Phosphate	175	750	0.633	2.0	6×10^4
3-layers High Phosphate	525	750	2.100	2.0	6×10^4

SAMPLING AND ANALYSIS:

Chlorophyll-*a*

Alongside with the dilution with fresh media, samplings for chl-*a* were conducted every second day in both experiments. Prior to sampling, gentle mixing of the bottles was performed in order to ensure sample homogeneity.

I used two different methods of chl-*a* analysis, a freeze drying method (*Experiment 1 & 2*) and traditional filtering (*Experiment 2*). The freeze drying method was originally designed for freshwater studies and allows for evaporating the water without damaging the algae (Hagerthey et al. 2006), but has never been applied to salt water samples before. In *Experiment 2* I also tested the reliability of this method against that of the traditional chl-*a* filtration method in experiment 2 (Arar and Collins 1997).

For the freeze drying method 1ml of sample was stored in labeled Eppendorf tubes and frozen for further analysis. A volume of 320µl from each thawed sample was then placed in 96-well plates and freeze dried. After the water had dried out, I added 320µl of ethanol (96%) to each well and allowed pigments to extract for 24h (Thrane J.E., Unpublished data). The next day I measured the emitted fluorescence using an excitation wavelength of 430nm and a detection wavelength of 675nm in a plate reader (Biotek, Synergy MX). For calculating the amount of chlorophyll-*a* in my samples I calibrated the fluorescence emittance using a 1 µg/L chlorophyll-*a* standard, of which I had diluted down to 1/264 of the initial concentration.

For *Experiment 2,* the rest of the sample (14ml) was sieved through a 50µm pore-size mesh and later filtered through glass microfiber filters (2.5cm Whatman GF/A). The filter was placed in labeled Eppendorf tubes (1.5ml) and frozen for later pigment extraction. The filters were defrosted and extracted over night in 1ml of ethanol (96%) directly in the Eppendorf tubes. I then placed 320µl of each sample in 96-well plates and measured the emitted fluorescence as described above (430, 675nm).

Finally I tested the correlation between the two methods, using all the available samples from *Experiment 2.* The reason that I chose to use a technique like freeze drying is that it is overall quicker, easier and cheaper than the filtration.

Rotifers

Upon the finalisation of *Experiment 1* , abundance of rotifers and their eggs were counted under a stereoscope. All rotifers in the bottles were counted without subsampling. I screened the contents of each flask through a 32μm sieve, resuspended the rotifers in counting chambers and counted them under a stereoscope.

In *Experiment 2* I also monitored the population of the rotifers along during the experiment. At each regular dilution with fresh media, the 14ml of sample were screened through a 50μm pore-size mesh to capture rotifers within the sampled amount of medium. After carefully rinsing of the sieve into Petri dishes, the sample was killed with a drop of Lugol's solution and the abundance of rotifers counted immediately. At the end of the experiment I subsampled and counted a volume of 50 ml for rotifers and eggs. In cases when the counts were low (<400) I subsampled another 50 ml. Counts were then corrected to the total bottle volume.

STATISTICAL ANALYSIS:

The abundance data on rotifers, their eggs and their algal prey were suitable for time series analysis with linear models, which can account for both temporal autocorrelation within individual units, as well as the partitioning of variance between units and treatment groups. The outcome of this analysis is estimates of means as well as auto- and cross-covariances of abundances of rotifers and algae in relation to surface to volume ratio of the growth habitat.

The effects of the treatment factors (surface to volume ratio and phosphate level) were tested with a two-way analysis of variance (2-way - ANOVA). I used the rotifer abundance in the end of both experiments and alga growth data for the last 4 days as dependent variables. All data used for modeling was logarithmically transformed for easier interpretation of the results.

Ratio dependence implies proportionality between prey and predator, while prey dependence does not. Therefore I used graphic modelling to test for ratio dependence by a regression model formulated as:

$$\log(N) = a + b\log(P) + c\,(S{:}V) \qquad (Eq.\ 1)$$

Where P is rotifer abundance, N is algal biomass (chl-a values) and $S{:}V$ is the surface to volume ratio. Under pure prey dependence b will be identically zero such that N is independent of P and only a function of S:V ratio, while b = 1 would correspond to pure ratio dependence. In other words, we can potentially discriminate between prey and ratio dependence from whether the confidence interval of the regression slope of log(N) predicted by log(P) includes 0 or 1.

All graphs and figures are produced using R software (R Core Team 2015)

Results

Rotifers

Experiment 1 Rotifer abundance

For Experiment 1 the surface to volume ratio in 3 and 5 layered bottles was calculated to 2.09 and 1.91 respectively. Since the difference between those two was minute I conjoined the two treatment levels. Therefore the non-layered flasks will be characterized as low surface to volume treatments and the 3 and 5 layered flasks as high surface to volume treatments.

As seen in Figure 7 the carrying capacity of the prey plays an important role on the population abundance for rotifers. The 0.5μM phosphate treatments had a very small amount of rotifers, ranging from 0.9 to 1.3 individuals×ml^{-1} in the low surface to volume replicates, while in the high surface to volume replicates the number of rotifers was bigger, ranging from 1.09 to 2.5 individuals×ml^{-1} with the distribution being rather symmetric. As for the 1 μM phosphate treatments the rotifers number increased but so did the variation among the replicates. For the non-layered replicates the abundance ranged between 3.4 and 5.3 individuals×ml^{-1}. Despite the variation, the distribution of the data was symmetric with no outliers. In the high surface to volume replicates an increase on the rotifer abundance was noticed as their numbers ranged from 3.7 up to 6.3 individuals×ml^{-1}. In the 2 μM treatments, the variation was also noticeable. In the low surface to volume replicates the rotifers varied from 6.5 to 9.52 individuals×ml^{-1}, while in the high surface to volume replicates the numbers varied between 12.2 and 18.2 individuals×ml^{-1}. It should be noted that the distribution for data of the high surface to volume replicates the numbers treatments is slightly skewed upwards (Figure 7).

Egg production at the end of the experiment depended on nutrient concentrations ($P < 0.001$), (Fig. 8). In the low surface to volume and poor phosphate treatments egg concentrations ranged from 0.078 to 0.124 eggs×ml^{-1} or 0.04 to 0.06 eggs×female^{-1}, while the high surface to volume flasks contained 0.09 to 0.12 eggs×ml^{-1} or in terms of fecundity 0.04 to 0.06 eggs×female^{-1} (Fig. 9). In the medium phosphate treatments, the amount of eggs was higher and the egg abundance rose as the surface to volume ratio increased. The fecundity values ranged between 0.05 and 0.07 eggs×female^{-1} for low surface to volume treatments and 0.06 and 0.08 eggs×female^{-1} for high surface to volume ratio flasks. Finally in the phosphate rich treatments the number of eggs increased significantly, while the replicates showed a big variation as well. In the non-layered flasks I measured 0.64 to 1.19 eggs×ml^{-1} or 0.07 to 0.15 eggs×female^{-1}, in the layered flasks the numbers varied from 1.16 to 2.33 eggs×ml^{-1} or 0.07 to 0.12 eggs×female^{-1}, and in the 5-layered treatments the lowest egg count was 1.3 eggs×ml^{-1} but the highest reached 2.55 eggs×ml^{-1} or 0.08 to 0.12 eggs×female^{-1}.

Observing the boxplots in Figure 8 one can notice that distribution of data for the low surface to volume treatments are skewed downwards, while the respective ones for high surface to volume, they are skewed upwards. In Figure 9 the fecundity of rotifers for *Experiment 1* can be observed. However, statistical analysis revealed a small but significant contribution of the increasing surface to volume ratio into the egg to rotifer ratio or fecundity (Pr=0.0107, R^2=0.4, n=45).

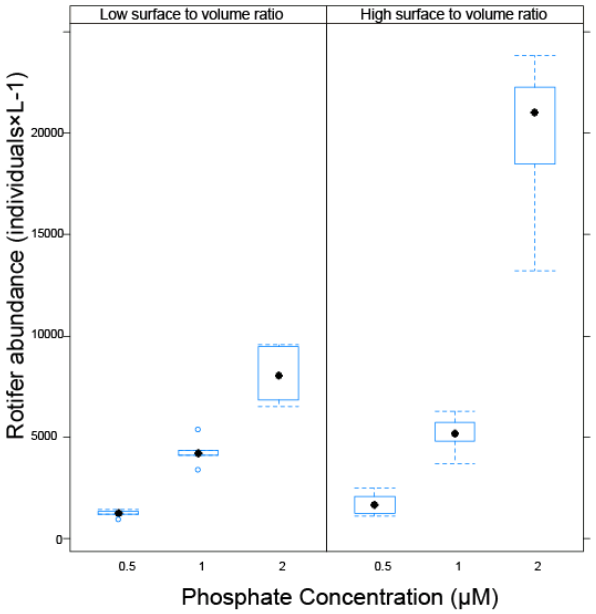

Figure 6 Rotifer abundance in *Experiment 1*.

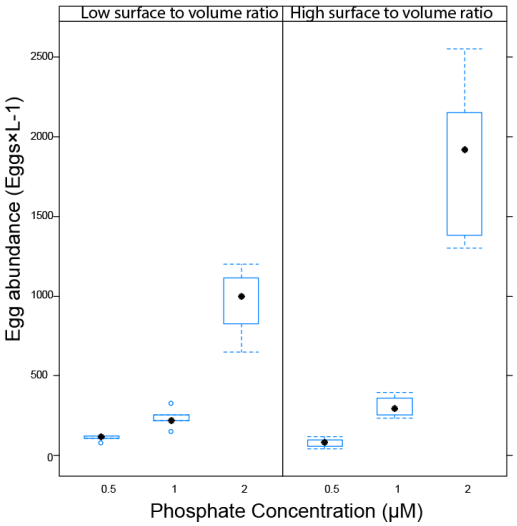

Figure 7 Egg abundance in *Experiment 1*

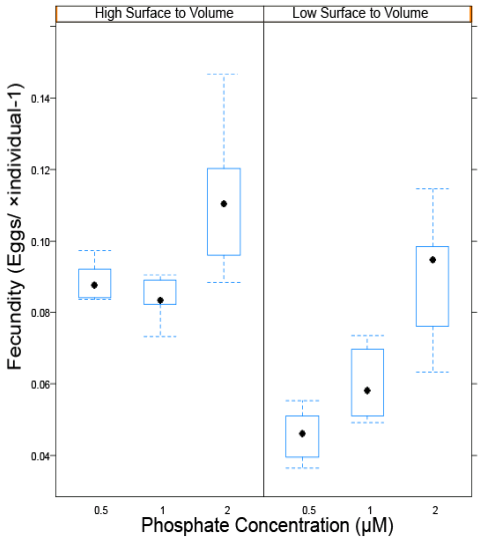

Figure 8 Fecundity data on *Experiment 1*

*Boxes represent 25th and 75th percentiles, the black dot is the median , and whiskers represent the 5th and 95th percentiles

Firstly, in the poor phosphate treatments (0.5µM), the low surface to volume replicates had a good amount of rotifers per liter, averaging at 7.6 individuals×ml^{-1}. As it can be observed in Figure 10, the distribution is skewed upwards, and two outlying points one at 5.2 and another at 9.1 individuals×ml^{-1}. However, in the high surface to volume replicates the number of rotifers was smaller, ranging from 5.7 to 7.8 individuals×ml^{-1} with the distribution slightly skewed downwards. Secondly, in the rich phosphate treatments (2µM) treatments, the variation was also big. In the low surface to volume replicates the rotifers varied from 6.5 to 9.45 individuals×ml^{-1}. Finally in high surface to volume replicates the numbers rose from 12.2 to 16.7 individuals×ml^{-1}. In phosphate rich treatments we see the distributions skewing downwards for low surface to volume and symmetric for high surface to volume, while for the high surface to volume ratio the point at 16.7 individuals×ml^{-1} is the highest recorded for *Experiment 2*.

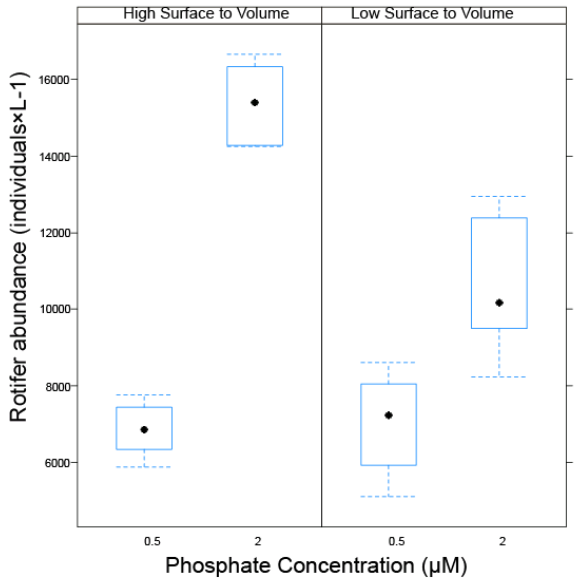

Figure 9 Rotifer abundance in *Experiment 2*

For *Experiment 2* the number of eggs varied considerably between phosphate treatments (Figure 10). For phosphate poor treatments the fecundity values rated between 0.14 to 0.2 in low surface to volume replicates, with the average being close to 0.15, and for the high surface to volume replicates from 0.1 to 0.14, the average being close to 0.14 while there is an outlying point at 0.18 as seen in Figure 11. For phosphate rich treatments, the average fecundity values did not vary much along the surface to volume gradient for low surface to volume it was 0.22 with symmetric distribution for low surface to volume replicates and 0.22 with the distribution skewed upwards for the high surface to volume ratio. The small variation among the three treatments, excluded the poor phosphate but high surface to volume, could hint towards a ratio dependent hypothesis. However there was no statistically significant contribution of the increased surface availability observed towards the production of eggs on rotifers.

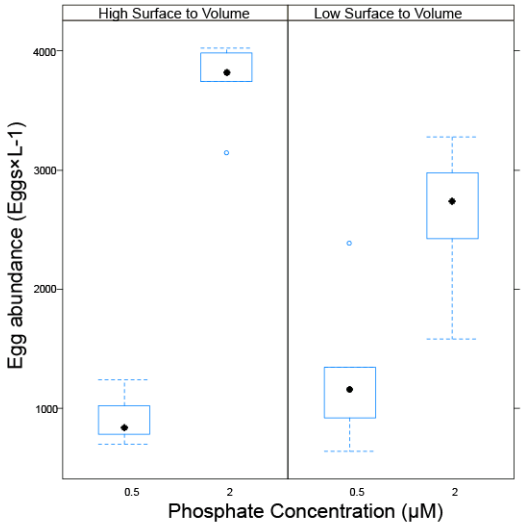

Figure 10 Egg abundance in *Experiment 2*

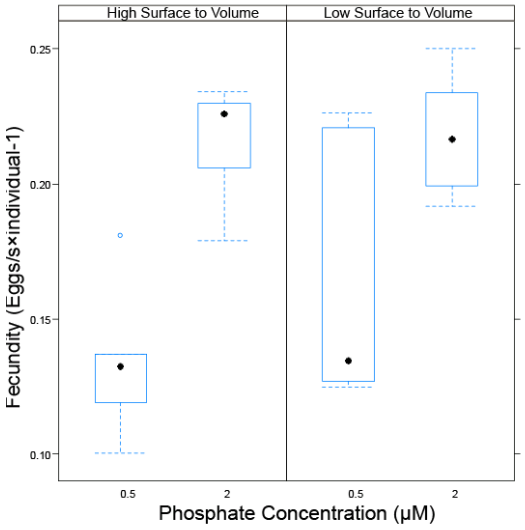

Figure 11 Fecundity in *Experiment 2*

Algal biomass

Experiment 1 Algal biomass

The development of the algal biomass followed a similar pattern in all the treatments. In the beginning the microalgae grew without a lag phase. Peak algal biomasses varied between phosphate concentrations, such that treatments richer in phosphate showed increased carrying capacity. The peak of the microalgae bloom was reached during the first four to eight days. Subsequently the algal prey biomass declined to very low levels after day 12, due to the grazing by the increasing numbers of predating rotifers. This decline is characterized as the post-bloom period. As an example of this rotifer growth I present the development on a replicate of a 3-layered flask on a 2 µM phosphate concentration (Figure 13). It was also the one with the highest biomass peak observed in the whole experiment, on the sixth day the chl-*a* level was measured to 0.052 $\mu g \times L^{-1}$. The graphs for the rest of the treatments can be found in Figures 19, 20, 21 in the Appendix.

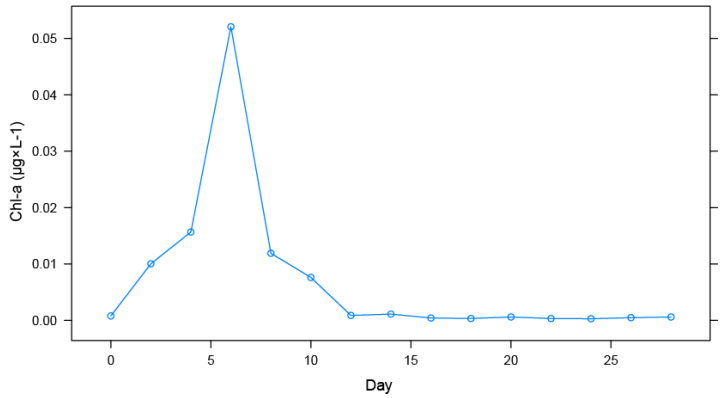

Figure 12 Example of algal biomass development on a replicate in Experiment 1 (2µM phosphate concentration, high surface to volume replicate)

The rotifers must have grown following the algal bloom, and a stable state of algal biomass was reached, which presumably continued until the final day of *Experiment 1*. Nonetheless, the temporal development of chl-*a* was generally consistent between treatments and replicates, indicating that rotifers grazed down the initial algal bloom until an equilibrium between growth and loss was established

in the treatments (See Appendix, Figures 19, 20, 21). However, rotifers were only measured at the beginning and end of the experiment.

Experiment 2 Algal biomass

In *Experiment 2* the development of the algal biomass did not follow a similar pattern in all the replicates. In the poor phosphate concentration (0.5μM) treatments the algae developed differently. Growth is observed in the first 10 days and the maximum peak was reached in days 8-10 in most replicates in low surface to volume replicates after the high peak was reached (days 8-10) a steady decline was observed while the population of rotifers slowly increased, as the treatments were reaching their carrying capacity. In fact, in one of the low phosphate treatments the rotifer population increased so much in the last few days of the experiment that it reached a high of 8.4 individuals×ml^{-1} and it can be observed at Figure 8. Especially for the high surface to volume replicates, the algae continued growing and remained at high levels, as the population of rotifers did not seem to have the same increase as in the other treatments (Figure 13).

The carrying capacity was bigger on the treatments with 2μM phosphate concentration. Initially in those phosphate rich treatments, the microalgae grew exponentially and peaked in the first 4-6 days. In Figure 13 it is can be easily observed that once the population of rotifers begins to increase, the algal biomass is starting to decline. In 2μM phosphate concentration treatments, the maximum peaks observed in chl-*a* produced by the algae in day 4 was 0.255 μg×L^{-1} in low surface to volume replicate and 0.179 μg×L^{-1} in a high surface to volume replicate (Figure 13). Respective data rotifer abundance and chl-*a* pigments extracted with freeze drying can be found in Figure 22 in the Appendix.

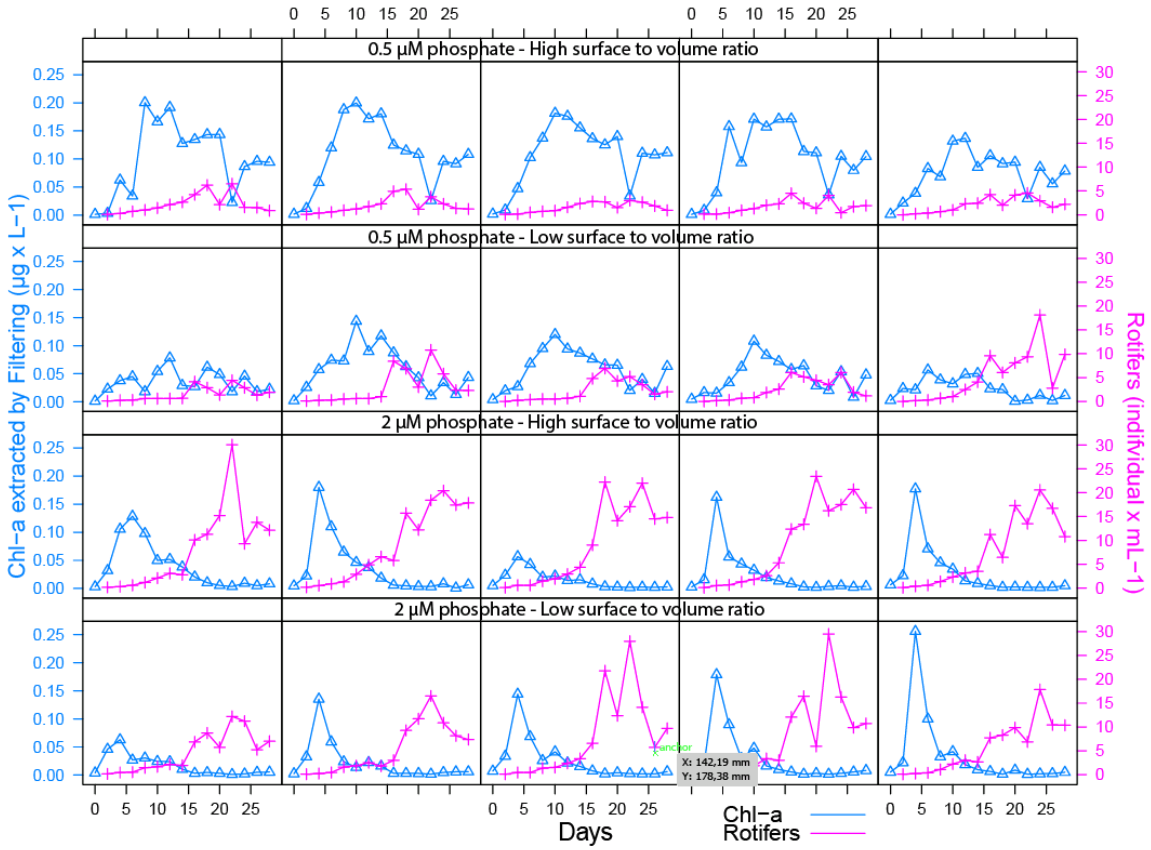

Figure 13 Rotifer abundance and algal development (measured by filtering) in Experiment 2.

Chl-a yields correlated strongly between the two extraction methods in *Experiment 2* (R^2 = 0.93, F=133.13, *Pr*<0.001, n=20) (Figure 14). Regardless the day of the sampling, chl-*a* yields were significantly greater for samples that were freeze-dried compared with material that had only been filtered before extraction. When compared with fresh/frozen samples extracted with 96% ethanol, more than 90% more chlorophyll was extracted when the sample was freeze-dried. A thorough view comparing the two methods for every sampling day is presented in Figure 24, in the Appendix.

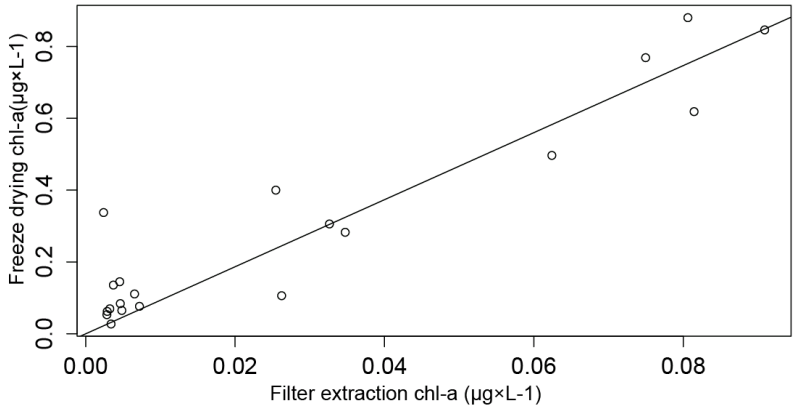

Figure 14 Data for Chl-a extracted with Freeze Drying or Filtering (on the average value of the last 4 sampling days, R^2 = 0.93)

I investigated the impact of the surface factor on the abundance of rotifers, by choosing a linear model (*Eq. 1*) and using the data from a steady state post bloom period of *Experiment 1* (last four sampling days) as well as the rotifer abundance count in the end of the experiment. A small but significant effect of surface to volume (*S:V*) was observed in the factorial ANOVA, after the grouping of the 3 and 5-layered flask treatments in *Experiment 1* (F=2.9612, *Pr* =0.09283, n=45). Model predictions (*Eq. 1*) upon chlorophyll-a concentrations (mg×L^{-1}) and rotifer densities (individuals×L^{-1}) can be viewed at Figure 15 (R^2=0.54, p=1.055×10^{-7}, n=45).

Contrariwise, a significant relationship was not observed through the results of factorial ANOVA upon egg abundance and the surface to volume ratio (F=1.6308, p>0.1, n=45). The above used model simplifies to common regression lines with positive slopes (Figure 16A). However, when tested, fecundity is affected significantly by the increase on surface to volume ratio (Pr=0,0232, R^2=0.40, p=8.393×10^{-5}, n=45) (Figure 16 B).

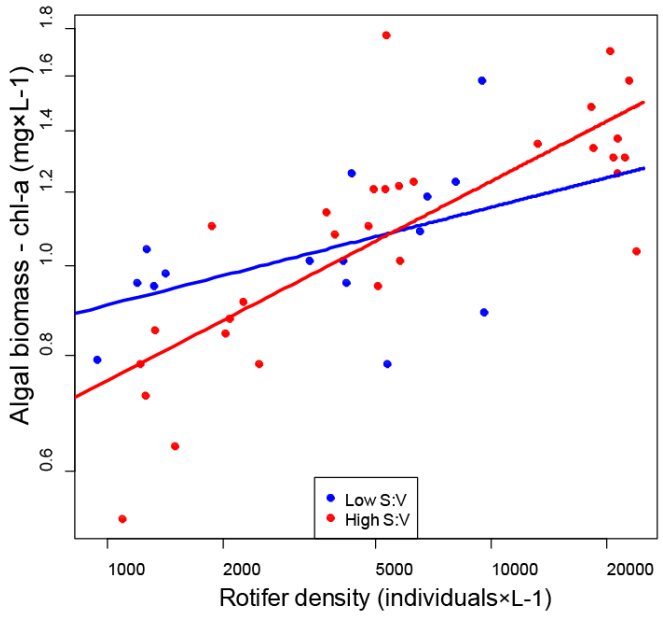

Figure 15 Rotifer density on algal biomass data results from Experiment 1. The algal biomass data are an average of the last 4 days Chl-a counts

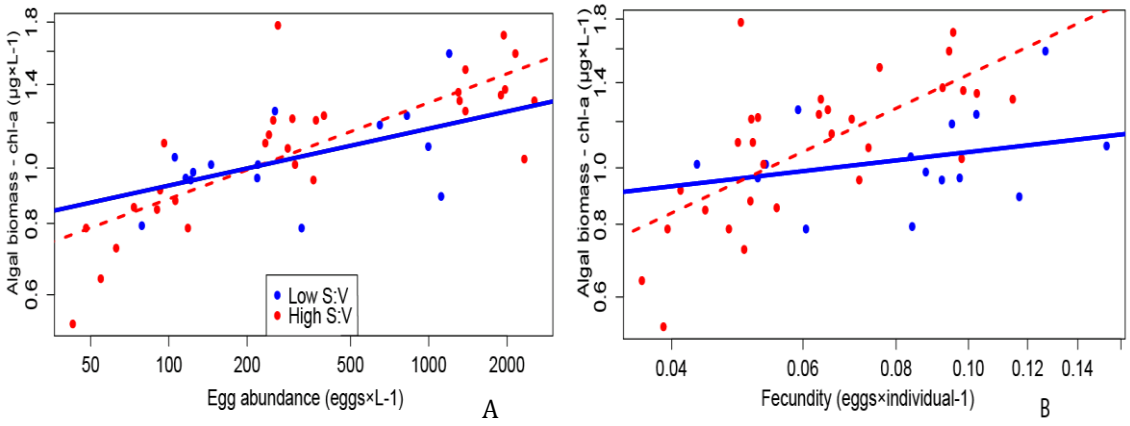

Figure 16 A) Egg density on algal biomass B)Fecundity on algal biomass, (Experiment 1)

In addition data from *Experiment 2* provide a better defined interpretation. Using data from the filter extraction method, the relationships between chlorophyll-α concentration and rotifer abundance can be better explained by the model (*Eq. 1*) (R^2=0.94, p=9.873×10^{-10}, n=20) (Figure 17). The effect of the phosphate concentration gradient seems to be significant (p<0.001), while the ANOVA test confirmed the significant interaction between surface to volume ratio increase and rotifer abundance (F=8.8745, *Pr*=0.008859, n=20).

However when the model (*Eq. 1*) is applied to data extracted from the freeze drying method its explanatory value decreases (R^2=0.64, p=0.0007205, n=20). Additionally, a small change in the surface significance values of is observed, (F=5.0218, *Pr*=0.0395675, n=20) but the result still remains statistically significant (Figure 18). However, a slope can be observed at the high surface to volume prediction line, which is absent in the prediction made using data from filter extraction.

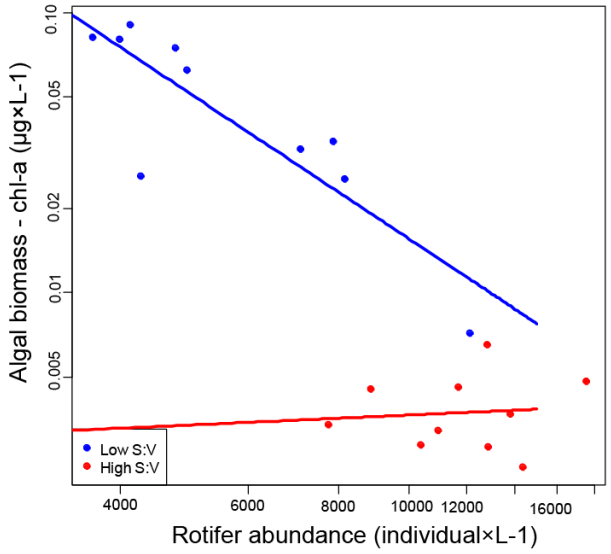

Figure 17 Rotifer density on algal biomass data results from Experiment 2. The algal biomass data are an average of the last 4 days filtered samples.

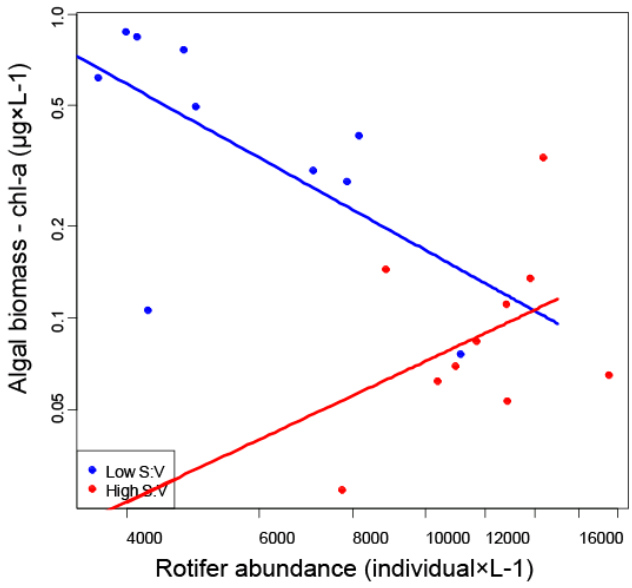

Figure 18 Rotifer density on algal biomass data results from Experiment 2. The algal biomass data are an average of the last 4 days freeze dryed samples.

Discussion

This study showed that the algal biomass was positively affected by increased nutrient levels, which led to greater rotifer abundance. There is also an interaction of the surface to volume factor that appears as a potential parameter for rotifer growth in line with Vadstein, Olsen et al. (2012). Before discussing those main results, I will compare and discuss the pigment extraction methods.

Comparing pigment extraction methods

Several studies have introduced freeze drying as a method used for extracting pigments from mostly freshwater samples and a few applied it as a pretreatment for marine benthic samples (Preston 1997, Wright et al. 2005). My application of freeze drying on salt water samples in 96-well plates is unique. On the other hand filter extraction is the classical method used for all types of samples of the water column. The results from the two pigment extraction methods I used in *Experiment 2* correlated strongly in patterns of algal biomass development (Figure 14). However, the pigment yields when freeze drying was used as a pretreatment of sample were very much higher than the pigment yields extracted by filtering (Appendix, Figure 23). Several studies have shown that pigment extracted via freeze drying is considerably more potent than those extracted via filtering, but this potency difference varies from 6 to 13% and in some cases up to 86% (Hagerthey et al. 2006, Szymczak - Żyła et al. 2008). Nonetheless, they use different types of algae as biomass indicators and it is presumed that different algal taxa can yield different extraction efficiency, depending on their theca structure and thickness (Pápista et al. 2002).

Another fact that should be taken into account is that the samples destined for filter extraction were screened through 50μm pore-size mesh so that rotifers could be counted. The average size of *Tetraselmis sp.* is (10μm long x 14μm wide), however green algae often form chains or aggregates in order to avoid predation or sinking (Guillard and Kilham 1977), these aggregates are often of greater size.

Potentially, a reason that filter extracted chl-*a* values were smaller than the ones coming from freeze drying, could be that a substantial part of algal cells aggregated on the mesh and thus leading to fewer pigments in the sample.

For arriving at a safe conclusion for supporting my models, both methods should be taken into consideration, but it should be taken into account that filter extraction is the traditional standard method for algal biomass determination. Despite the fact that freeze drying is not commonly used, I have confidence in its use as a pigment extraction method as its advantages in lower cost and workload are notable. Yet its confidence levels need to be further tested against the traditional methods.

Algal biomass

With intention of quantifying the magnitude of the variation I used a phosphate nutrient gradient, which is frequently used to monitor the development of primary producers under the stress of predation (Watson et al. 1992). In *Experiment 1* the prediction that higher phosphate concentration controlled which type of treatment would grow more abundant in algal biomass was rather satisfied. Moreover, the trend toward highest density of algae biomass values registered was a direct consequence of the difference on the phosphate levels in my treatments. Higher algal growth rates were measured on the richer phosphate concentration ($p< 0.001$).

A variety of conceptually important questions about picking the correct period for taking down my setup in *Experiment 2* may be raised, but after examining data from *Experiment 1* (Appendix, Figure 19, 20, 21) I was convinced that all my treatments would have reached their steady phase at least 20 days after the beginning of the experiment. That did not seem to happen for all treatments in *Experiment 2* (Figure 13). Moreover, a degree of caution must be used when evaluating *Experiment 1* and *Experiment 2* since differences in algal biomass development may make the results extracted rather inconclusive.

In spite of comparing growth patterns for algal biomass, among the different levels of nutrients, the moment the growth peak was reached and its size differed, respectively to each of the nutrient levels. Phosphate poor treatments did grow slower and the chl-*a* counts peaked at much lower points than the phosphate rich treatments. In this way, facing the high proportion of variation in the algal biomass the rotifer abundance varied respectively among treatments.

Rotifer abundance and fecundity as a function of surface habitat

Remaining on the attached surface probably represents an energy-saving strategy used by rotifers (Vadstein et al. 2012). On this basis, surfaces seem to have another impact on a variety of prey-predator systems, besides the one as refuge from predators (Jessup et al. 2005). Thus, development among rotifer assemblies and their dependence on the variation of habitats might largely have been underestimated in field samples. Many species of zooplankton conduct semi-benthic life styles in the pelagic, thus profiting on for example aggregates (Koski et al. 2005). Their concentration is higher on aggregates than in surrounding water (Steinberg et al. 1994). Rotifers of the species *Branchionus plicatilis* can deliberately switch from a free-swimming to an attached state depending on the food concentration levels in their habitat. When food concentration levels are high enough, surfaces are comparatively more important, as rotifers can benefit from this attached state and maximize their net growth.

However, I did not investigate the exact way or the level of how much surfaces maximize net growth in rotifers. According to Vadstein et al. (2012) the profitability of surface attachment is twofold. Firstly, attachment in rotifers reduces the energy requirements of swimming, and the energy loss due to viscous drag (Fenchel 1987). Secondly, filter feeding while the rotifer is attached provides a better conversion efficiency of prey consumed to biomass than in free swimming rotifers (Epp and Lewis Jr 1984). As such, the relationship between surface attachment and prey consumptions has many aspects to be considered.

In Figure 6 one can observe that there is increased rotifer abundance with increased nutrients along with increasing surface to volume ratio in the treatments. Especially for Experiment 1 (Figure 6) it is obvious how the increase in phosphate concentration increases the rotifer abundance. It can also be observed that for treatments poor in phosphate (0.5 μM) there is a very small difference in the rotifer abundance among replicates, while for phosphate rich treatments, the variation among replicates is much more noticeable. In contrast, in *Experiment 2* the average rotifer abundance in the phosphate poor treatments was higher in low surface to volume ratio than in high surface to volume ratio (Figure 9). It should be taken into consideration the fact that those treatments may not yet have reached their steady state (Figure 13). The reason for this result thus remains unclear. It may be related to the starting conditions with fewer algae in *Experiment 2*.

However, in *Experiment 2*, the behavior of the rotifer populations in the low phosphate concentration but high surface to volume ratio is puzzling. At such limiting prey concentration it is possible that rotifers do not prefer the attached state(Figures 9, 13). Possibly the layers in the flasks worked as a barrier for them being able to swim looking for food, as the compartments of the layered flask are joined only in the two edges, while the rotifers in non-layered flasks had more free space to look for algae to graze on when in search for food.

Typical clearance rates for the rotifer *Branchionus plicatilis* range between 3.4-6.9 $\mu l \times ind^{-1} \times hr^{-1}$ and have been related to the physiological status of the rotifers (Korstad et al. 1989). Assuming maximum clearance rates and my data on rotifer abundance in the end of the experiments, rotifers in my experiments would be capable of clearing the flask within a day during food limited conditions (such as in the low P treatments). Residence times of rotifers on walls have been measured as>20 minutes for rotifers (Vadstein et al. 2012). Possibly surface attachment is preferred when prey availability is high, while when there is scarcity of prey rotifers may have to put an effort in swimming for food search. This difference could be attributed to the fact that increased surface to volume effects are more apparent when higher nutrient concentration, which is higher carrying capacity of prey.

Similarly, the egg production of rotifers was examined in both my experiments. In *Experiment 1* fecundity was significant correlated between with the amount of surface habitat (*Pr*=0.0107) although it did not explain a big part of the variation (R^2=0.4) (Figure 8). The data from experiment 2 did not come to verify that. Rotifers require a sufficient food consumption so as to commence with sexual reproduction and resting egg production (Serra et al. 2004). Attachment to surfaces may provide some benefit over but it is not clearly statistically indicated in my experiments.

Modelling the prey-predator interaction

My results of the algal biomass data and rotifer abundance in the end for both experiments varied in different ways (Appendix, Figure 25, 26, 27). The classical prey-dependent functional response predictions dictate that steady state prey biomass should not vary with carrying capacity. This was an indication that the functional response could be explained by a ratio-dependent model. To determine whether the models produced reasonable results, I compared the empirical values from the algal biomass data in the end of the experiments with the predictions deriving from the model where the only explanatory value is surface to volume ratio.

Whereas defining the shape of a functional or numerical response in grazing zooplankton in general, the role of surfaces available has received little consideration for either its effects to the rotifer population or the potential uses of them. However in the model I used S:V the only explanatory factor. To evaluate the results after *Experiment 1*, I decided to run *Experiment 2*, with a slightly altered design, leaving some parameters out and also using filter extraction as a source for collecting data for the algal biomass.

Considering the experiments separately, the slope for the predicting lines partially suggests ratio-dependence in *Experiment 1* (Figure 16). The response of the low surface to volume predicting line though perplexes the outcome of this model

prediction, as it should in theory be parallel with the one for high surface to volume. Another way to interpret it would be that the surface to volume availability contributes more in cases where the carrying capacity of prey is higher. However, taking a look at varying algal biomass levels (Appendix, Figure 29), and rotifers abundance in the end of the experiment, one can observe a proportional increase in along the nutrient and surface to volume gradient, which points towards the ratio dependency hypothesis (Arditi and Ginzburg 1989).

To determine whether *Experiment 1* produced reasonable results I compared it with the values for freeze drying and filter extraction for *Experiment 2*. A degree of caution must be used when comparing chl-*a* values directly, since differences in techniques employed may have a strong impact on results obtained. Although the scaling exponents matched well (Appendix, Figure 24), the result of those methods did not agree for the model predictions part. Furthermore, for *Experiment 2* the filter extraction data indicated a prey-dependent response for the high phosphate concentration treatments (Figure 17), which comes into disagreement with the predictions made by Vadstein, Olsen et al. (2012).

The reflection of a prey-dependence response could be due to that steady state in the treatments was not reached. Nevertheless, ratio-dependent effects are in most cases introduced when measuring the functional response in a longer time scale (Arditi et al. 1991), that is well into the steady state in systems like the one presented in this study. If instead we consider the data extracted via freeze drying (Figure 18), the slope of the prediction line could be contradicting the prey dependence hypothesis. It could potentially be attributed to some data being overexpressed by freeze drying. Also, the prey density dependent response hypothesis is supported by the data for algal biomass levels which remain steady carrying capacity increases (Appendix Figures 30, 31), excluding the low phosphate and high surface to volume replicates.

As seen as in the low phosphate concentration treatments in *Experiment 2*, an unexpected behavior of the algal biomass that grew in the high surface to volume flasks rendered the dynamics of those systems far from being characterized stable. Therefore, the model predicts negative density dependence, which is a reflection of the low rotifer abundance in those bottles (Figure 17). Negative density dependence is a recognized stabilizing factor, but this last possibility is rather inconsistent. In addition the mechanisms that produce such negative density dependence have not been studied well (McPeek 2012), which would make it very difficult to connect my experiments data to theoretical studies.

Further research on prey and ratio dependent prey-predator models on rotifers could reveal interesting dynamics such as deterministic extinction and existence of various attractors, surface attachment included. In addition to providing a plausible explanation of the use of submerged objects as profitable habitat for these animals both in fresh and salt water, these surface dynamics may offer a reasonable support to observations already made by researchers.

- My project is among the few studies designed to experimentally test the effects of available surfaces on predator biomass development. The results revealed a significant effect of available surfaces into rotifer population, which is amplified when there is an abundance of nutrients available.
- The ratio-dependent model was generally the most informative with regard to conclusions about rotifer biomass development under different surface to volume ratio for *Experiment 1*.
- Even when the dynamic described is apparently pointing to prey-dependent response in *Experiment 2*, the replicates had not reached a post-bloom steady state so conclusions should be drawn carefully. The effect of surfaces availability is obscure on circumstances of food limitation in my replicates.
- The study makes a prediction about the general mechanism that could explain the dependence on surface attachment along with prey availability. A variety of conceptually important questions about the circumstances where rotifers pick to switch to an attached-state strategy remains unresolved.
- The consequences of temporary attachment of rotifers-predators to surfaces thus seem to be an interesting topic for further ecological and evolutionary studies. It could be of quantitative importance for *Branchionus plicatilis* in mass cultures or its native habitat as well.
- When a preparatory method is applied before pigment extraction from salt-water samples, caution should be exerted in choosing the procedure, as it may have significant implications for the precision and accuracy. The findings in this study could provide a basis for additional investigation.

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Experiment 1 graphs on algal biomass development over time

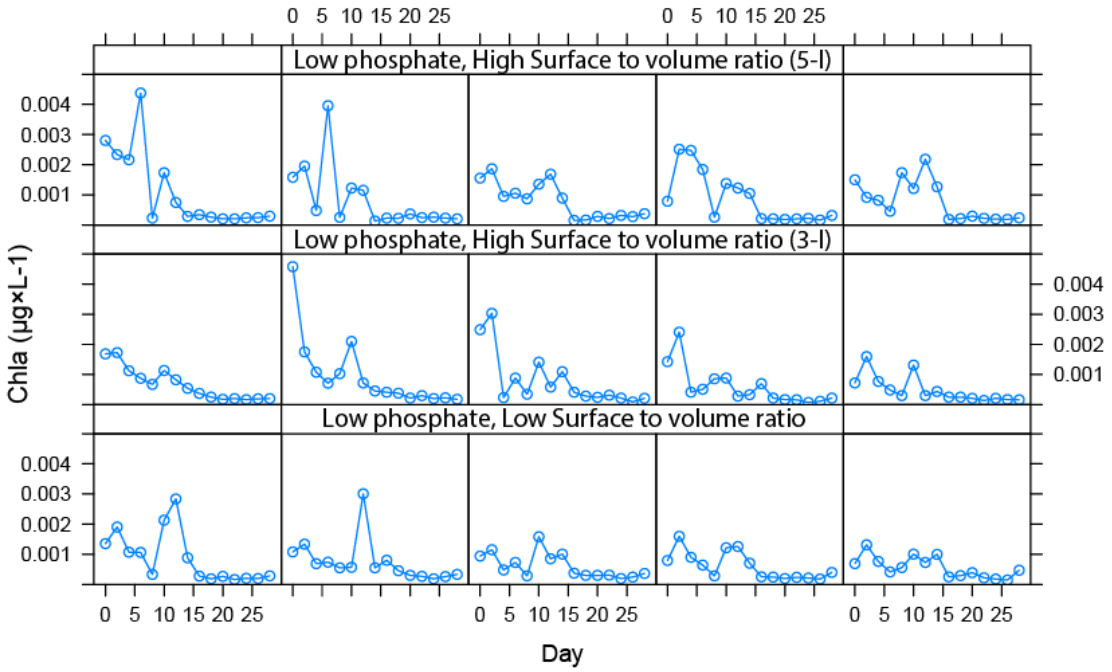

Figure 19 Algal biomass growth on low phosphate concentration treatments (0.5µM) in Experiment 1. Replicates of low S:V non layered, of high S:V 3 layered (3-l) and 5 layered flasks (5-l)

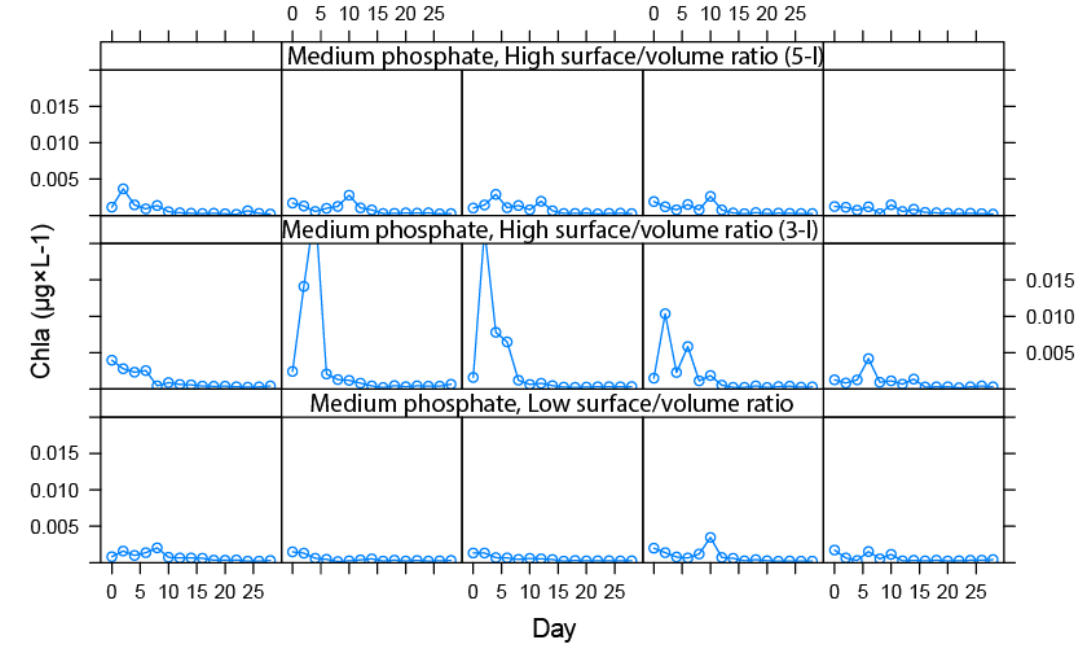

FIGURE 20 Algal biomass growth on medium phosphate concentration treatments (1µM) in Experiment 1

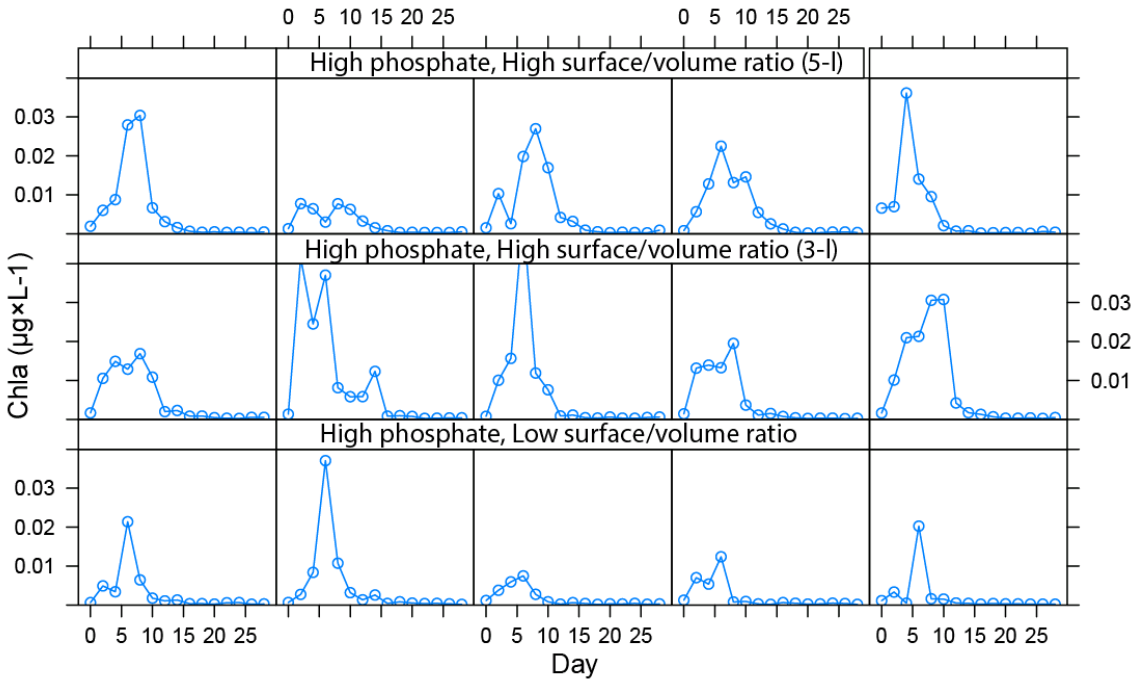

Figure 21 Algal biomass growth on high concentration phosphate treatments (2µM) in Experiment 1. Replicates of low S:V non layered, of high S:V 3 layered (3-l) and 5 layered flasks (5-l)

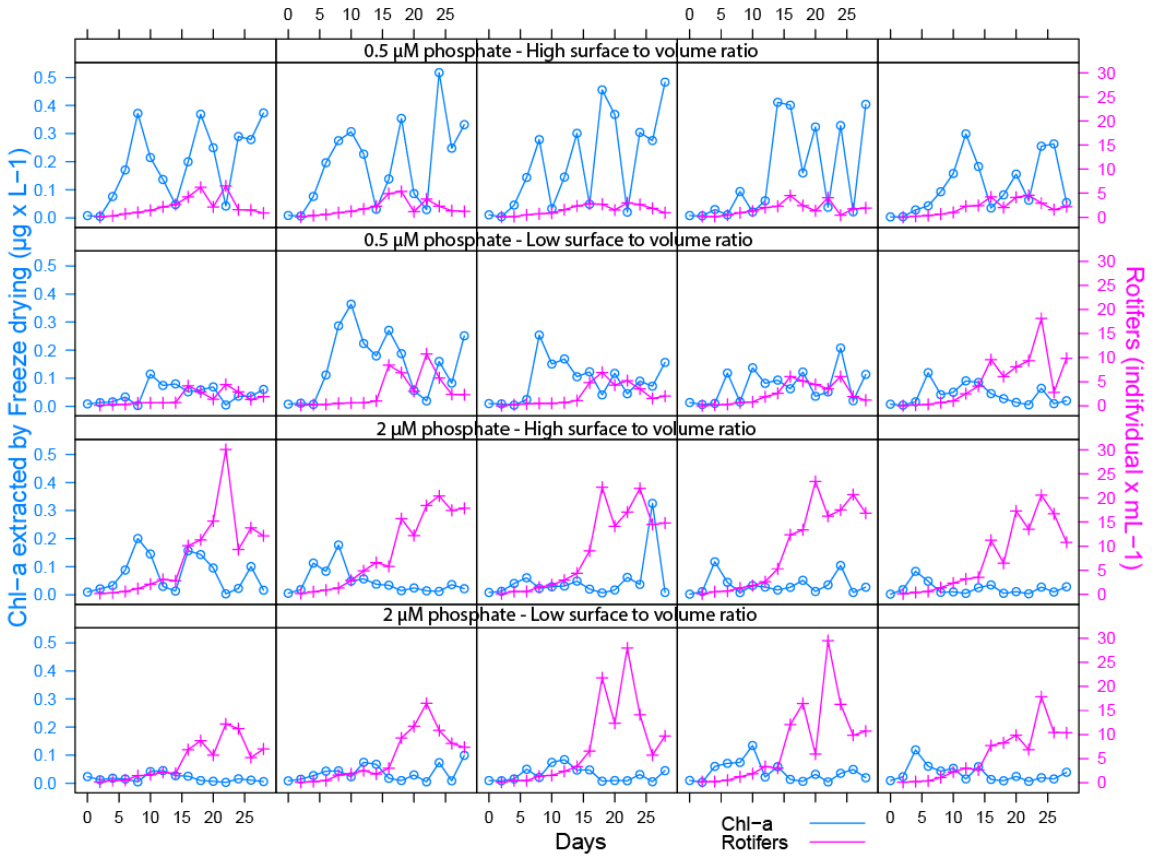

Figure 22 Rotifer growth and algal biomass (freeze-drying) plotted together. (Experiment 2)

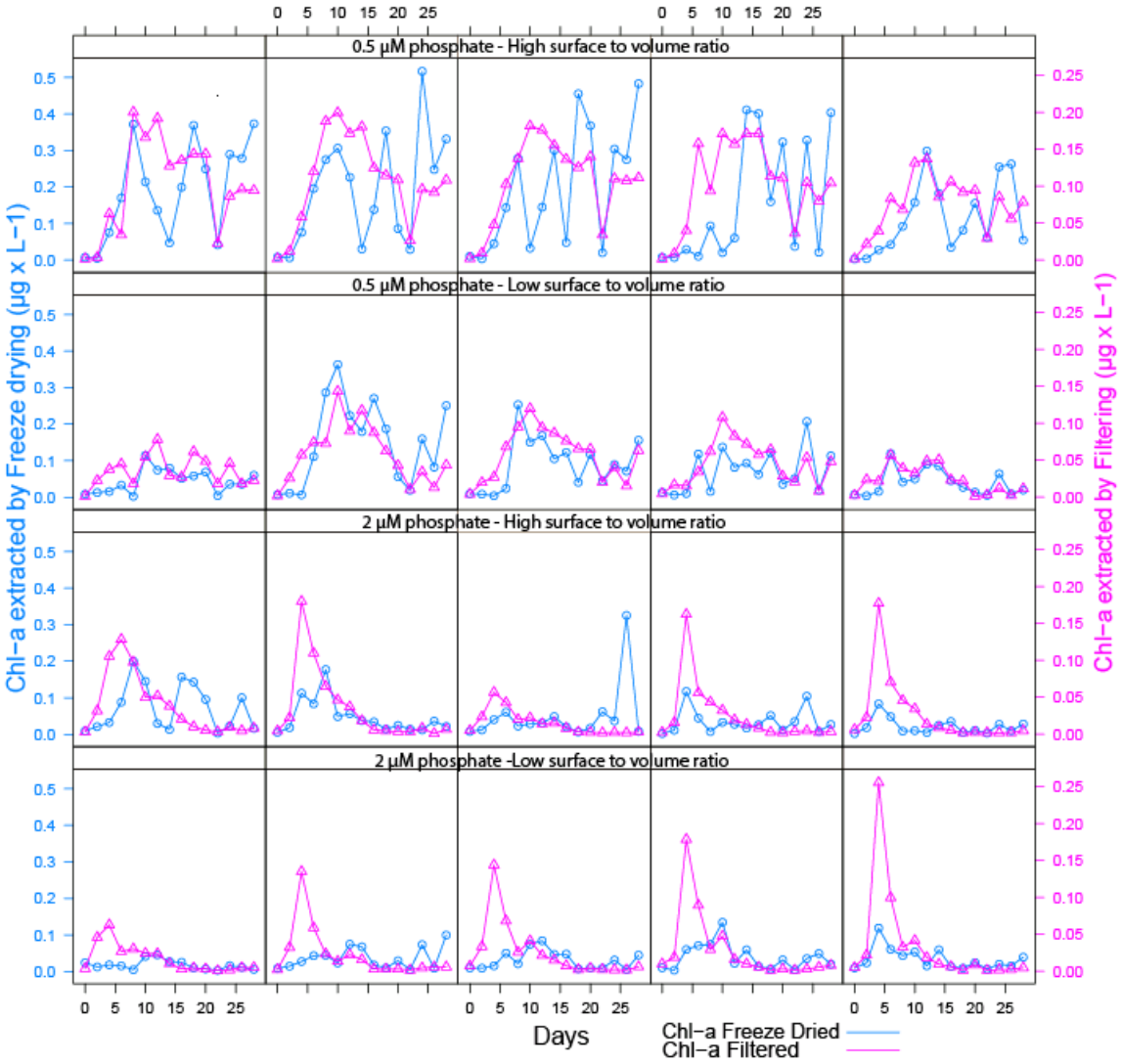

Figure 23 Algal biomass measured both by filtering and freeze drying plotted together. (Experiment 2)

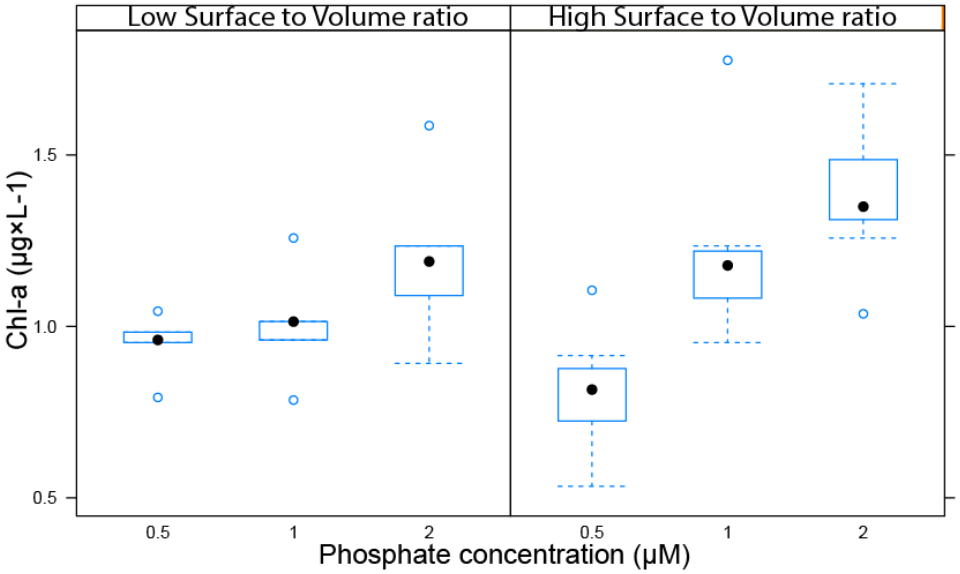

Figure 24 Chl-*a* levels in the end of Experiment 1

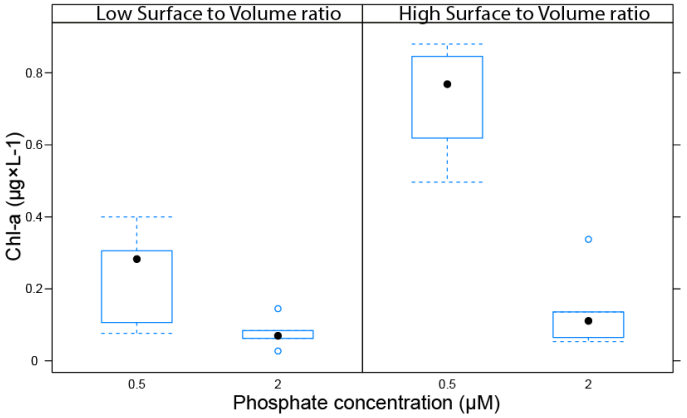

Figure 25 Chl-*a* levels in the end of Experiment 2. Freeze drying.

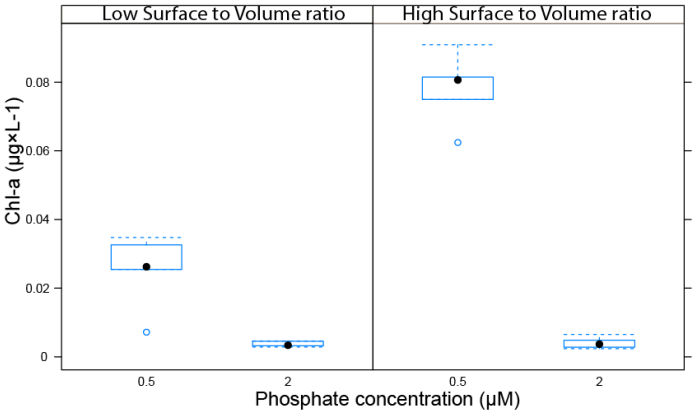

Figure 26 Chl-*a* levels in the end of Experiment 2. Filter extraction.